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CCL5 and cytokine expression in the rat brain: Differential modulation by chronic morphine and morphine withdrawal

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ABSTRACT

Opioids have been shown to influence the immune system and to promote the expression of pro-inflammatory cytokines in the central nervous system. However, recent data have shown that activation of opioid receptors increases the expression and release of the neuroprotective chemokine CCL5 from astrocytes *in vitro*. To further define the interaction between CCL5 and inflammation in response to opioids, we have examined the effect of chronic morphine and morphine withdrawal on the *in vivo* expression of CCL5 as well as of pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Rats undergoing a chronic morphine paradigm (10 mg/kg increasing to 30 mg/kg, twice a day for 5 days) showed a twofold increase of CCL5 protein and mRNA within the cortex and striatum. No changes were observed in the levels of IL-1β and TNF-α. Naltrexone blocked the effect of morphine. A chronic morphine paradigm with no escalating doses (10 mg/kg, twice a day) did not alter CCL5 levels compared to saline-treated animals. On the contrary, rats undergoing spontaneous morphine withdrawal exhibited lower levels of CCL5 within the cortex as well as increased levels of pro-inflammatory cytokines and Iba-1 positive cells than saline-treated rats. Overall, these data suggest that morphine withdrawal may promote cytokines and other inflammatory responses that have the potential of exacerbating neuronal damage.

1. Introduction

The adverse effects of opioids such as heroin and morphine on the adaptive and innate immune systems have been recognized [reviewed in Hutchinson et al. (2011), Roy et al. (2011)]. Less is known about opioid-induced changes in central nervous system (CNS) immune responses. Morphine has been shown to promote the expression of pro-inflammatory cytokines and chemokines in the CNS (Johnston et al., 2004; Schwarz et al., 2013). Cytokines and chemokines are soluble polypeptides that were discovered for their ability to modulate immune responses as well as cell migration. In the CNS, these polypeptides are synthetized and released mostly by glial cells, which are considered the immune competent cells of the CNS as well as crucial components of synaptic plasticity (Huang et al., 2000; Ransohoff and Brown, 2012). Therefore, opioids, through cytokines and chemokines, could contribute to pathological states or participate in the maintenance of healthy neurons. On the other hand, morphine-mediated increase of cytokines/chemokines appears to be more pronounced in brain areas that participate in the neuronal network involved in opiate abuse/reward, including the nucleus accumbens (Schwarz et al., 2011). Moreover, in recent years, several lines of independent investigations have shown that the immune system may play a role in the behavior outcome following opioid administration [reviewed in Coller and Hutchinson (2012)]. Thus, re-evaluating whether a given cytokine or chemokine is enhanced in response to an inflammatory pathological state or to activation of a brain circuitry involved with drug abuse is crucial for a better understanding of opioid action and immune responses.

There are a number of cytokines and chemokines in the brain (Cartier et al., 2005). Chemokines and their receptors are often involved with the progression of neurodegenerative diseases. However, some of these molecules exhibit a profile similar to that of neuromodulators (Adler and Rogers, 2005; Rostene et al., 2007) or behave like neurotrophic factors. For instance the chemokine CCL5, formerly known as regulated on activation normal T-cell expressed and secreted (RANTES), was discovered for its ability to attract and activate mononuclear phagocytes, as well as several other leukocyte types, to sites of inflammation/injury. However, CCL5's main receptor CCR5 is expressed in the CNS (Avdoshina et al.,

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2011; Westmoreland et al., 2002). CCL5 exerts neurotrophic (Bolin et al., 1998) and neuroprotective activity against various neurotoxins including glutamate, beta-amyloid, HIV and nitric oxide (Bruno et al., 2000; Kaul et al., 2007; Tripathy et al., 2010). Injection of CCL5 into the periaqueductal grey blocks the antinociceptive effect of opioid agonists (Chen et al., 2007). Moreover, chronic morphine promotes the expression and release of CCL5 from primary cortical astrocytes (Avdoshina et al., 2010) or astrocytes from the nucleus accumbens and ventral tegmental area in morphine-dependent rats (Hutchinson et al., 2009). Thus, this chemokine could participate in modulation of pain in response to opioids or play a role in opioid dependence.

Cessation of morphine use leads to withdrawal. It has been established that withdrawal can be detrimental to immunosuppression leading to susceptibility to outside pathogens. For example, opioid withdrawal can reduce the levels of circulating lymphocytes in morphine dependent macaques (Weed and Steward, 2005). Moreover, opioid withdrawal can affect cytokine production in blood cells (Das et al., 2011; Desjardins et al., 2008) or in brain (Liu et al., 2011). Thus, the profile of chemokine/cytokine expression in rats that are morphine-dependent or undergoing withdrawal could be different. This study aimed to elucidate the *in vivo* effects of chronic morphine treatment, in comparison to morphine withdrawal on the expression of CCL5 and pro-inflammatory cytokines that could be involved in the mechanisms of neurotoxicity or drug addiction.

2. Materials and methods

2.1. Animals

Three month old male Sprague–Dawley rats (Charles River, Germantown, MD) were acclimated a minimum of one week prior to conducting experiments. Homozygous CCL5 (CCL5-/-) mice (B6.129P2-Cccl5 tm1Hso/J) and wild type C57BL/6 J mice were purchased from the Jackson Laboratory (Famington, CT). Mice were viable, normal in size and did not display any gross physical or behavioral abnormalities. Animals were housed under standard conditions, two per cage, with food and water available *ad libitum*, and were maintained on a 12-h light/dark cycle for the duration of the treatment protocols. All studies were carried out following the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and approved by Georgetown University Animal Care and Use Committee.

2.2. Animal treatments

Morphine sulfate was received from the National Institutes of Drug Abuse, (NIH, Division of Neuroscience & Behavioral Research, Research Triangle Park, NC). (-)- Naltrexone hydrochloride (NTX) was purchased from Sigma, St Louis, MO. All drugs were diluted in isotonic and filtered sterilized saline. Drugs doses are reported as free base. To cause morphine tolerance and dependence rats were treated s.c. with escalating doses of morphine from 10 mg/kg to 30 mg/kg b.i.d over the course of five days (Table 1). Rats were sacrificed 2 h after the last injection. A group of rats received escalating doses of morphine concomitantly to NTX (10 mg/kg, s.c.) to block the analgesic effect of morphine. The efficacy of chronic NTX in preventing morphine induced analgesia was determined by the radiant tail-flick method according to an established protocol (D'Amour and Smith, 1941). In brief, animals were placed on the tail-flick apparatus (Socrel DS20, Milan, Italy) with their tails smoothed into the tail grove. The radiant heat intensity was calibrated to obtain average latency values of 4 s. A cut off time of 10 s was used to avoid tissue damage. Baseline was established for all animals before treatment paradigms to reduce the novelty effect of the behavioral task.

To cause morphine withdrawal, animals were treated with increasing dosages of morphine (10 mg/kg to 30 mg/kg) b.i.d. over the course of five days as described above, after which, morphine treatment was interrupted and spontaneous withdrawal observed for 60 h. Withdrawal symptoms included diarrhea, weight loss, piloerection and irritability, compared to control animals.

After the treatments, animals were anesthetized with a mixture of ketamine/xylazine (80 mg/kg and 20 mg/kg, respectively) and intracardially perfused with ice cold phosphate buffered saline (PBS). Whole brains were quickly removed for biochemical or immunohistochemical analyses. For biochemical analyses, frontal cortex, striatum, hippocampus and cerebellum were dissected on ice. For immunohistochemistry, whole brain were removed and post-fixed in 4% paraformaldehyde (PFA) overnight followed by 30% sucrose.

2.3. Enzyme-Linked Immunosorbant Assay (ELISA)

Brain lysates were prepared as previously described (Bachis et al., 2010). CCL5, IL-1β, and TNF-α levels were determined using the DuoSet ELISA Development System Kits (R&D, Minneapolis, MN), according to the manufacturer's instructions. The content of total proteins in each sample was determined by bicinchoninic acid assay (Thermo Scientific Inc., Rockford, IL).

2.4. Western blot analysis

Brain areas were homogenized in RIPA buffer (Millipore, Billerica, MA) containing protease-phosphatase inhibitors (Roche Applied Science, Indianapolis, IN) by sonication and protein elution at 4 °C. Proteins were separated in a NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and transferred to a PVDF membrane using the iBlot device (Invitrogen). Membranes were blocked with 5% milk in PBS and 0.05% Tween and probed with rabbit polyclonal anti-CCR5 antibody (1 μg/ml, Abcam, Cambridge, MA). Membranes were stripped and reprobed with mouse monoclonal anti β-actin antibody (1:10,000, Sigma–Aldrich, St. Louis, MO) as a loading control. Immune complexes were detected by the corresponding secondary antibody and chemiluminescence reagent (Thermo Fisher Scientific Inc.). The intensity of immunoreactive bands was quantified using Quantity One® 1-D (Bio-Rad Laboratories, Inc., Hercules, CA) and expressed in arbitrary units (A.U.) after normalization to β-actin immunoreactivity.

2.5. Semi-quantitative reverse transcriptase polymerase chain reaction (qPCR)

Total mRNA was extracted using the RNeasy Plus Mini Kit according to manufacturer's specifications (Qiagen, Valencia, CA). qPCR for CCL5 was performed using primers that displayed linear amplification as previously described (Avdoshina et al., 2010). Ribosomal protein L13 (RPL13) and TATA box binding protein were used as housekeeping genes to normalize for total mRNA in each sample. Primers for housekeeping gene were obtained from RealTimePrimers.com. For RPL13 were 5-GTGAGGGCATCAACATTTCT-3, 5-CATCCGCTTTTTCTTGTCAT-3 (forward, reverse); For TATA box, 5-CGATAACCCAGAAAGTCGAA-3, 5-AGATGGGAATTCCAGGAGTCTTTTCCAGCAAGTCAATCC-3, (forward, reverse). The specificity of the qPCR was determined by the analysis of the dissociation curves. The 7900HT Fast Real-Time PCR System (Life Technologies Corp, Carlsbad, CA) was used. Data were analyzed using SDS v2.3 software (Life Technologies).